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DESCRIPTION

K01-0509 substance and process for production thereof

5 Technical Field

The present invention relates to K01-0509 substance which inhibits type III secretion mechanism of bacteria and a process for production thereof. More particularly the present invention pertains to K01-0509 substance comprising K01-0509-A1 substance
10 and/or K01-0509-A2 substance which are useful as a remedy or preventive for infectious disease.

Background Art

The type III secretion mechanism, which is a function for
15 releasing bacterial pathogenic factor to the extracellular field, has been reported to be highly conserved, for example, in bacteria of genus Salmonella, genus Yersinia, genus Pseudomonas, Shigella, enteropathogenic E. coli (hereinafter sometimes designates as EPEC), enterohemorrhagic E. coli and
20 genus Bordetella (Microbiology and Molecular Biology Reviews, June, 1998, p. 381).

It has been reported that bacteria maintaining the type III secretion mechanism hereinabove released the pathogenic factor into the extracellular field through the secretion
25 mechanism and a part of the released pathogenic factor was transferred into the host cell, and the pathogenic factor transferred into the host cell was largely involved in the pathogenicity of bacteria (Microbiology and Molecular Biology Reviews, June, 1998, p. 382 ff.).

On the other hand, it was demonstrated that EPEC strain defective in type III secretion system (hereinafter sometimes designates as type III secretion protein) lost the pathogenicity in the infectious experiments using rabbits (J. Exp. Med. 188(10), 1907-1916, 1998, November 16) and the infectious experiment using human volunteers (Infection and Immunity, June 2000, p. 3689-3695). From these facts, substances inhibiting the type III secretion system and the function of the secretion protein are expected to exhibit effects as a remedy or preventive remedy for infectious disease having new ideas without killing bacteria but making the pathogenicity disappear.

Disclosure of the invention

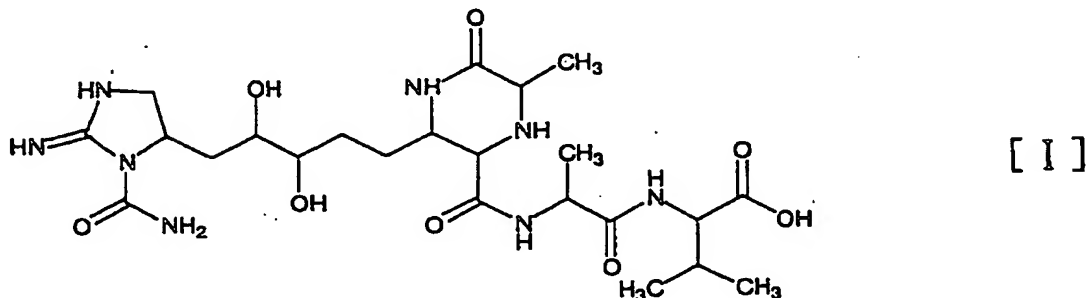
In such circumstances, problem to be solved by the present invention is to find out new antiinfectious disease agents which allow to disappear the pathogenicity of pathogenic bacteria.

In order to solve such problems hereinabove described, we have continued studies on microbial metabolites, and found that substances having inhibitory activities against the type III secretion system were produced in the cultured medium of a newly isolated strain, designated as K01-0509, from soil sample collected in Amami-Oshima. Further, we have found that substances represented by the chemical structure of the formula [I] and [II] hereinbelow as a result of isolation and purification of the active principle from the cultured mass showing inhibitory activities against the type III secretion system. Since substances having such chemical structures were not known, these substances were designated as K01-0509-A1 substance and K01-0509-A2 substance, and were totally designated as K01-0509

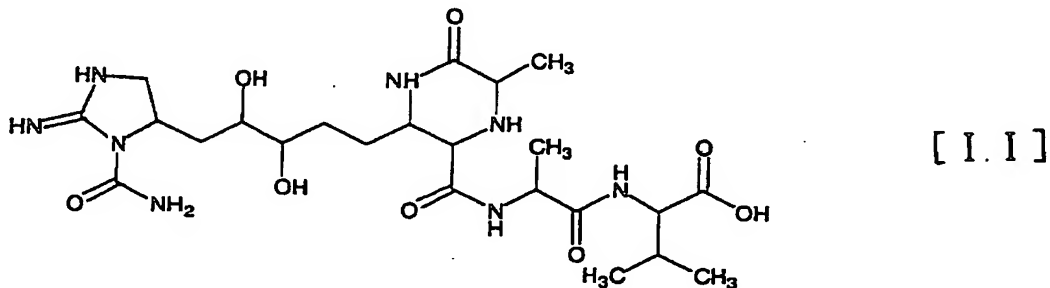
substance.

The present invention has been completed by such knowledge.

An aspect of the present invention is to provide
5 K01-0509-A1 substance represented by the following formula [I];

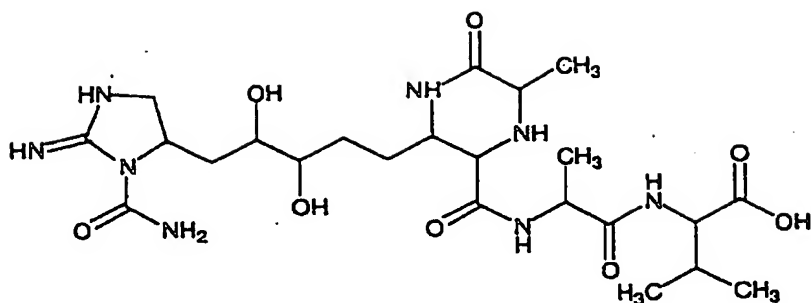


An aspect of the present invention is to provide
K01-0509-A2 substance (a stereoisomer of A1 substance) represented by the following formula [II];



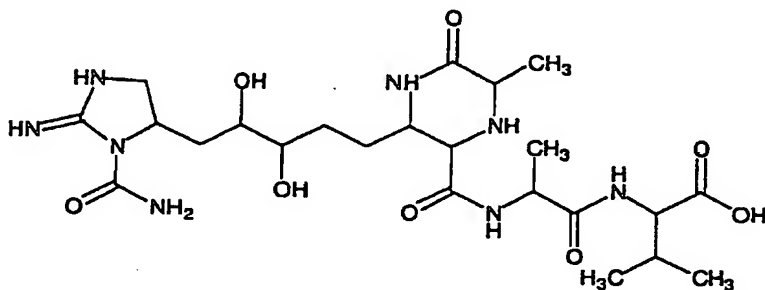
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Further aspect of the present invention is to provide
K01-0509A substance consisting of K01-0509-A1 substance
represented by the following formula [I];



[I]

and K01-0509-A2 substance represented by the following formula
[II];



[I . I]

5 Another aspect of the present invention is to provide a process for production of K01-0509-A1 substance comprising culturing microorganism belonging to genus Streptomyces and having ability to produce K01-0509-A1 substance, accumulating K01-0509-A1 substance in a cultured medium and isolating
10 K01-0509-A1 substance from the cultured mass.

More further aspect of the present invention is to provide a process for production of K01-0509-A2 substance comprising culturing microorganism belonging to genus Streptomyces and having ability to produce K01-0509-A2 substance, accumulating
15 K01-0509-A2 substance in a cultured medium and isolating K01-0509-A2 substance from the cultured mass.

Further aspect of the present invention is to provide a process for production of a composition consisting of K01-0509-A1 substance and K01-0509-A2 substance comprising

culturing microorganism belonging to genus Streptomyces and having ability to produce K01-0509-A1 substance and K01-0509-A2 substance, accumulating K01-0509-A1 substance and K01-0509-A2 substance in a cultured medium and isolating K01-0509-A1
5 substance and K01-0509-A2 substance from the cultured mass.

Further aspect of the present invention is to provide a process for production of K01-0509-A1 substance wherein a microorganism belonging to genus Streptomyces and having ability to produce K99-05278-A1 substance is Streptomyces sp.
10 K01-0509 FERM BP-08504.

Further aspect of the present invention is to provide a process for production of K01-0509-A2 substance wherein a microorganism belonging to genus Streptomyces and having ability to produce K99-05278-A2 substance is Streptomyces sp.
15 K01-0509 FERM BP-08504.

Further aspect of the present invention is to provide a process for production of a composition consisting of K01-0509-A1 substance and K01-0509-A2 substance wherein a microorganism belonging to genus Streptomyces and having
20 ability to produce K99-05278-A1 substance and K01-0509-A2 substance is Streptomyces sp. K01-0509 FERM BP-08504.

Further aspect of the present invention is to provide a microorganism which is Streptomyces sp. K01-0509 FERM BP-08504.

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The microorganism having ability to produce K01-0509-A1 substance and K01-0509-A2 substance or a composition thereof represented by the formula [I] and [II] hereinbefore (hereinafter designates as "K01-0509 substance producing micro-

organism") belongs to genus Streptomyces, and, for example, Streptomyces sp. K01-0509, which was newly isolated by the present inventors, is the most preferable strain used in the present invention.

5 Taxonomical properties of Streptomyces sp. K01-0509 of the present invention are as follows.

(I) Morphological properties

Vegetative mycelia grow well on various agar media and no fragmentation is observed. Aerial mycelia are abundantly grown
10 on yeast-malt extract agar medium and glycerol-asparagine agar medium, and exhibit white to grayish color. On microscopic observation, chains of more than 20 spores are observed on the aerial mycelia, and the morphological form is linear chains and size of spore is about $0.6 - 0.8 \times 1.0 - 1.8 \mu\text{m}$ with cylindrical
15 form. Surface of the spore is smooth. Sclerotia, sporangia and zoospores are not observed.

(II) Culture properties on various media

Culture properties of the producing strain of the present invention determined by the method of E. B Shirling and D.
20 Gottlieb (International Journal of Systematic Bacteriology, 16: 313, 1966) are shown in the following. Color tone was determined referring to Color Harmony Manual, 4th Ed. (Container Corporation of America, Chicago, 1958) as a standard color, and color name as well as attached code number in the parenthesis.
25 Unless otherwise noted, results are observation of cultures at 27°C for 2 weeks on various media.

Culture properties

Sucrose-nitrate agar medium	
Growth	good growth, light amber (3ic)
Reverse side	light amber (3ic)
Aerial mycelium	moderate epiphytic, white (a) - gray (h)
Soluble pigment	none

Glucose-asparagine agar medium	
Growth	good growth, pearl pink (3ca)
Reverse side	pearl pink (3ca)
Aerial mycelium	none
Soluble pigment	none

Glycerol-asparagine agar medium (ISP)	
Growth	good growth, light amber (3ic)
Reverse side	light amber (3ic)
Aerial mycelium	abundantly epiphytic, white (a) - gray (f)
Soluble pigment	none

Starch-inorganic salt agar medium (ISP)	
Growth	good growth, light amber (3ic)
Reverse side	light tan (3gc) - light amber (3ic)
Aerial mycelium	moderate epiphytic, white (a) - gray (h)
Soluble pigment	none

Tyrosine agar medium (ISP)	
Growth	good growth, bamboo (2gc)
Reverse side	bamboo (2gc)
Aerial mycelium	moderately epiphytic, white (a)
Soluble pigment	none

Oatmeal agar medium (ISP)	
Growth	good growth, light amber (3ic)

Reverse side	light tan (3gc) - orange rust (4pe)
Aerialmycelium	moderately epiphytic, white (a) - dark cobalt gray (2ih)
Soluble pigment	none
Yeast-malt extract agar medium (ISP)	
Growth	good growth, amber (3lc)
Reverse side	light amber (3ic) - dark luggage tan (4pg)
Aerial mycelium	abundantly epiphytic, white (a)- gray (g)
Soluble pigment	none
Nutrient agar medium	
Growth	good growth, pearl pink (3ca)
Reverse side	pearl pink (3ca)
Aerial mycelium	none
Soluble pigment	none
Peptone-yeast-iron agar medium (ISP)	
Growth	good growth, bamboo (2fb)
Reverse side	bamboo (2fb)
Aerial mycelium	poorly epiphytic, light ivory (2ca)
Soluble pigment	none
Glucose-nitrate agar medium	
Growth	moderate growth, bright (3ia)
Reverse side	orange (4la)
Aerial mycelium	none
Soluble pigment	none
Glycerol-calcium malate agar medium	
Growth	good growth, bamboo (2fb)

Reverse side	pearl pink (3ca)
Aerial mycelium	poorly epiphytic, white (a)
Soluble pigment	none
Glucose-peptone agar medium	
Growth	good growth, light ivory (2ca)
Reverse side	pearl pink (3ca)
Aerial mycelium	none
Soluble pigment	none

(III) Physiological properties

(1) Formation of melanin pigment

(a) Tyrosine agar negative

(b) Peptone-yeast-iron agar medium negative

5 (c) Tryptone-yeast liquid positive

(d) Simple gelatin medium (21 - 23°C) false positive

(2) Nitrate reduction negative

(3) Liquefaction of gelatin (21 - 23°C) (simple gelatin medium)
negative

10 (4) Starch hydrolysis positive

(5) Coagulation of defatted milk (37°C) positive

(6) Peptonization of defatted milk (37°C) positive

(7) Growth temperature 10 - 38°C

(8) Utilization of carbon sources (Pridham-Gottlieb agar
15 medium)

Utilize: D-glucose, L-arabinose

Not utilize: D-xylose, D-mannitol, L-rhamnose,
D-fructose, myo-inositol, raffinose,
melibiose, sucrose

(9) Decomposition of cellulose

negative

(IV) Composition of cell wall

2,6-diaminopimelic acid of cell wall is LL type. Main
5 menaquinone is MK-9(H₆) and MK-9 (H₈).

(V) Conclusion

Taxonomical properties of the strain of the present
invention are summarized as follows. 2,6-diaminopimelic acid
10 in the cell wall is LL type and main menaquinone is MK-9(H₆) and
MK-9 (H₈). Morphology of the spore chain is linear chain, forming
with long spore chains and smooth spore surface. Various
properties on the culture are exhibiting brown color tone
vegetative mycelia and white to grayish aerial mycelia.
15 Production of melanin pigment is observed in Tryptone-yeast
liquid.

According to results hereinabove, the present strain was
identified as the strain belonging to genus Streptomyces based
on descriptions in "Bergey's Manual of Systematic Bacteriology,"
20 Vol. 4, 1989".

The strain was deposited, based on Budapest Treaty on the
International Recognition of the Deposit of Microorganisms for
the Purposes of Patent Procedure, as Streptomyces sp. K01-0509
in International Patent Organism Depository, National Institute
25 of Advanced Industrial Science and Technology, AIST Tsukuba
Central 6, 1-1, Higashi 1-chome, Tsukuba-shi, Ibaraki-ken,
305-8566 Japan on October 6, 2003 as permanent depository number
FERM BP-08504.

The strain of Streptomyces sp. K01-0509 can be mentioned

as a preferable example of K05-0509 substance producing strain used in the present invention. However, since the morphological properties of microorganisms are generally very easily mutated and are not constant. Natural mutation or artificial mutation
5 generally performed by ultraviolet irradiation or chemical mutagens such as N-methyl-N'-nitro-N-nitrosoguanidine and ethyl methansulfonate, are well known. The strain belonging to genus Streptomyces and having ability to produce K01-0509 substance represented by the formula [I] and [II] hereinbefore,
10 including the artificial mutants as well as natural mutants or composition thereof, can be used in the present invention.

In a production of K01-0509 substance of the present invention, at first, K01-0509 substance producing strain belonging to genus Streptomyces is cultured in a preferable
15 medium. Nutrient sources preferable for production of K01-0509 substance of the present invention are assimilable carbon sources for microorganism, digestible nitrogen sources and, if necessary, inorganic salts. Examples of assimilable carbon sources are sugars such as glucose, fructose, maltose, lactose,
20 galactose, dextrin and starch, and plant oil such as soybean oil, etc. are used independently or in combination.

Examples of nitrogen sources are peptone, yeast extract, meat extract, soybean powder, cotton seed powder, corn steep liquor, malt extract, casein, amino acids, urea, ammonium salts
25 and nitrates are used independently or in combination. If necessary, salts such as phosphate, magnesium, calcium, sodium, potassium, heavy metallic salts such as iron, manganese, copper, cobalt or zinc, vitamins and substances suitable for production of K01-0509 substance are added.

In the liquid culture, if foaming occurs, antifoam agents such as liquid paraffin, animal oil, vegetable oil, silicone oil and surface active agent can preferably be added. The above culture can be performed by liquid or solid culture condition, 5 if the above nutrient sources are contained, and in general, the culture can preferably be performed using liquid culture medium, and in case of small production, the culture using flask is preferable.

10 In the large scale production using the large tank, in order to prevent delay of growth of microorganism in the production process, the production strain is inoculated and cultured initially in relatively small amount of culture medium, subsequently the cultured mass is transferred into the large tank and cultivation is preferably continued. In this case, 15 compositions of the medium used in the pre-culture and the medium used in the production culture can be identical or different if necessary.

In the culture under aeration spinning condition, conventional means, for example, agitation using propeller and 20 other mechanical stirring, rotation or shaking in fermenter, treating with pumping and blowing air can be applied. Air for aeration should be sterilized. Culturing temperature can be applied within ranges in the production of K01-0509 substance by K01-0509 substance producing strain, and the cultivation is 25 performed usually at 20 - 30°C, preferably at 27°C. Culturing pH is usually pH 5 - 8, preferably about pH 7. Culturing time depends on culturing condition and is usually for 3 days. The thus obtained accumulated K01-0509 substance in the cultured mass exists generally in cultured supernatant. Isolation of

K01-0509 substance from the cultured medium can be performed by methods used for isolation of metabolites from microbial cultured mass independently, repeatedly or in combination with any orders of the means.

5 Isolation and collection of K01-0509 substance can be performed by collecting from the cultured supernatant. The cultured supernatant is treated by the known method used for collection of water soluble substance, for example, chromatography such as adsorption chromatography, gel filtration
10 chromatography and high performance liquid chromatography, in combination or repetition thereof to isolate K01-0509 substance.

 Physicochemical properties of K01-0509 substance of the
15 present invention are explained hereinbelow.

1. K01-0509-A1 substance

(1) Nature: white powder

(2) Molecular formula: $C_{23}H_{40}N_8O_8$

HRFAB-MS (m/z) $[M+H]^+$

20 Calculated 557.3047, Found 557.3052

(3) Molecular weight: 556

FAB-MS (m/z) $[M+H]^+$ 557, $[M+Na]^+$ 579

(4) Ultraviolet absorption spectrum (in water): as shown in Fig.
1, terminal absorption

25 (5) Infrared absorption spectrum (KBr Tablet): as shown in Fig.
2, specific maximum absorption λ_{max} at 1564, 1682 cm^{-1} .

(6) Specific rotation: $[\alpha]_D^{27} = -5.00^\circ$ (c= 0.1, methanol)

(7) Solubility in solvent: soluble in water, dimethyl sulfoxide

(DMSO) and methanol,

: insoluble in acetonitrile, ethyl acetate, chloroform and acetone.

(8) Grouping for acidic, neutral and basic: Basic substance.

5 (9) Amino acid analysis: L-alanine and L-valine (1 : 1).

(10) ^1H nuclear magnetic resonance spectrum (in deuterium oxide) measured by using Varian NMR 400 MHz (Fig. 3). Chemical shifts of hydrogen (ppm) are: 0.92(3H), 0.92(3H), 1.39(3H), 1.44, 1.55(3H), 1.57, 1.71, 1.76, 1.77, 1.93, 2.11, 3.48, 3.61, 3.79, 10 3.97, 4.07, 4.17, 4.23, 4.27, 4.43 and 4.44.

(11) ^{13}C -nuclear magnetic resonance spectrum (in deuterium oxide) measured by using Varian NMR 100 MHz (Fig. 4). Chemical shifts of carbon (ppm) are: 18.2, 19.1, 20.1, 21.1, 29.6, 30.3, 32.5, 38.8, 52.2, 53.1, 53.3, 53.8, 54.0, 56.0, 62.4, 74.1, 76.7, 15 158.9, 158.9, 168.4, 172.1, 176.6 and 179.2.

As shown in above, as a result of detailed examination of various physico-chemical properties and spectral data of K01-0509-A1 substance, K01-0509-A1 substance was determined to have the chemical structure as shown in the formula [I].

20

2. K01-0509-A2 substance

(1) Nature: white powder

(2) Molecular formula: $\text{C}_{23}\text{H}_{40}\text{N}_8\text{O}_8$

HRFAB-MS (m/z) $[\text{M}+\text{H}]^+$

25 Calculated 557.3047, Found 557.3023

(3) Molecular weight: 556

FAB-MS (m/z) $[\text{M}+\text{H}]^+$ 557, $[\text{M}+\text{Na}]^+$ 579

(4) Ultraviolet absorption spectrum (in water): as shown in Fig. 5, terminal absorption

(5) Infrared absorption spectrum (KBr Tablet): as shown in Fig. 6, specific maximum absorption λ_{max} at 1564, 1682 cm^{-1} .

(6) Specific rotation: $[\alpha]_D^{26} = -7.4^\circ$ ($c = 0.1$, methanol)

5 (7) Solubility in solvent: soluble in water, dimethyl sulfoxide (DMSO) and methanol,
: insoluble in acetonitrile, ethyl acetate, chloroform and acetone.

(8) Grouping for acidic, neutral and basic: Basic substance.

10 (9) Amino acid analysis: L-alanine and L-valine (1 : 1).

(10) ^1H nuclear magnetic resonance spectrum (in deuterium oxide) measured by using Varian NMR 400 MHz (Fig. 7). Chemical shifts (ppm) are: 0.92(3H), 0.92(3H), 1.39(3H), 1.44, 1.53(3H), 1.57, 1.71, 1.76, 1.77, 1.93, 2.12, 3.48, 3.61, 3.79, 3.99, 4.07, 4.09,
15 4.23, 4.27, 4.44 and 4.50.

(11) ^{13}C -nuclear magnetic resonance spectrum (in deuterium oxide) measured by using Varian NMR 100 MHz (Fig. 8). Chemical shifts (ppm) are: 17.0, 19.2, 20.1, 21.1, 30.1, 30.4, 32.6, 38.8, 52.2, 53.2, 53.8, 54.0, 54.9, 59.8, 62.4, 74.2, 76.8, 158.9,
20 159.9, 168.3, 172.1, 176.6 and 179.0.

As shown in above, as a result of detailed examination of various physico-chemical properties and spectral data of K01-0509-A2 substance, K01-0509-A2 substance was determined to have the chemical structure as shown in the formula [II].

25 Biological properties of K01-0509 substance of the present invention are explained in detail hereinbelow. Inhibitory activity against the type III secretion system and antibacterial activity were assayed by the following methods.

(1) Assay of enteropathogenic E. coli type III secretion system

dependent hemolytic inhibitory activity

Test was performed according to method for detecting substances inhibiting the bacterial type III secretion system and function of secretory proteins thereof which was established by Omura et al. (WO 02/057760A1 and corresponding US Patent 6,586,200). One loopful enteropathogenic E. coli cesT defective strain (WO 02/057760A1) was inoculated in LB liquid medium (EB media 2.5%, Funakoshi Co. Ltd., Japan) (5 ml) and cultured at 37°C for 12 hours without shaking. Bacterial culture (1 %) was inoculated to M9 medium containing casamino acid (sodium dihydrogen phosphate 0.68% (Kanto Chemical Inc., Japan), potassium dihydrogen phosphate 0.3% (Wako Pure Chemical Industries Ltd., Japan), sodium chloride 0.05% (Kanto Chemical Inc., Japan), ammonium chloride 0.1% (Wako Pure Chemical Industries Ltd., Japan), glucose 0.4% (Wako Pure Chemical Industries Ltd., Japan), casamino acid 0.1% (Sanko Junyaku Co., Ltd., Japan) and magnesium sulfate 0.012% (Kanto Chemical Inc., Japan)) and cultured at 37°C for further 4 hours without shaking. Cultured liquid was centrifuged at 3500 rpm for 15 minutes to collect bacterial cell precipitation. The bacterial pellets were suspended in the fresh M9 medium (5 ml) to prepare the test bacterial liquid.

Erythrocytes was washed three times by centrifugation of the erythrocytes suspension, which was prepared by adding physiological saline (40 ml) to the sheep red blood cells (8 ml) (obtainable from Nippon Biological Materials Center, Japan), at 4°C at 2500 rpm for 5 minutes. Weight of the erythrocyte pellets was measured. M9 medium containing casamino acid (2 ml) was added to the erythrocytes pellet (1 g) by a ratio thereof

to prepare suspension of erythrocytes. The thus prepared E. coli suspension and erythrocyte suspension were mixed in equal quantities. The mixture (90 µl) was added to the 96-well microplate (Corning Inc., U.S.A.), to which the sample (5 µl) and M9 medium containing casamino acid (10 µl) were previously added. The plate was centrifuged at 1500 rpm for 10 minutes, and the hemolytic reaction was initiated at 37°C for 90 - 150 minutes. After the reaction, cooled PBS(-) (150 µl) (sodium chloride 0.8% (Kanto Chemical Inc., Japan), sodium dihydrogen phosphate 0.115% (Kanto Chemical Inc., Japan), potassium dihydrogen phosphate 0.02% (Wako Pure Chemical Industries Ltd., Japan) and potassium chloride 0.02% (Kanto Chemical Inc., Japan)) was added to prepare the suspension, and was centrifuged at 1500 rpm for 10 minutes. Supernatant (100 µl) obtained by the centrifugation was transferred into another 96-well microplate (Corning Inc., U.S.A.). Eluted hemoglobin was measured at 550 nm by using automatic microplate reader (Bio-Instruments Inc., U.S.A.). The hemolytic inhibitory activity is calculated by the following equation.

Inhibition rate (%) = $100 - [(A - C) / (B - C) \times 100]$

wherein

- A: data at 550 nm when added the sample
- B: data at 550 nm of the mixture of erythrocyte and enteropathogenic E. coli Cest defective strain alone
- C: data at 550 nm of erythrocytes alone

Results are as follows.

The drug concentration causing 50 % hemolytic inhibitory activity (IC₅₀) for K01-0509-A1 substance is 4.2 µg/ml and IC₅₀ for K01-0509-A2 substance is 3.2 µg/ml. Both substances were

demonstrated to inhibit type III secretion system.

(2) Assays of antibiotic activities for enteropathogenic E. coli and various test microorganisms by paper disc method

5 Test microorganisms used were enteropathogenic E. coli E2348/69 (wild strain), Bacillus subtilis ATCC 6633, Micrococcus luteus ATCC 9341, E. coli NIHJ, and Xanthomonas campestris pv. oryzae KB88. Medium used was nutrient agar medium (peptone 0.5% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), meat
10 extract 0.5% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan) and agar 0.8% (Shimizu Shokuhin K.K., Japan), adjusted at pH 7.0). Activity was evaluated by paper disc method (diameter 6 mm: Advantech Co. Ltd.), and inhibitory zone was measured after 24 hours cultivation.

15 As a result, no inhibition zone was shown in all test microorganisms for K01-0509-A1 at 10 µg/disc and K01-0509-A2 at 10 µg/disc.

 As described in detail, K01-0509 substance of the present invention can be expected as novel anti-infectious disease drug,
20 which inhibits selectively type III secretion system without exhibiting antibacterial activity.

Brief explanation of drawings

 Fig. 1 shows ultraviolet absorption spectrum of
25 K01-0509-A1 substance (in water).

 Fig. 2 shows infrared absorption spectrum of K01-0509-A1 substance (KBr tablet).

 Fig. 3 shows proton nuclear magnetic resonance spectrum of K01-0509-A1 substance (in deuterium oxide).

Fig. 4 shows carbon nuclear magnetic resonance spectrum of K01-0509-A1 substance (in deuterium oxide).

Fig. 5 shows ultraviolet absorption spectrum of K01-0509-A2 substance (in water).

5 Fig. 6 shows infrared absorption spectrum of K01-0509-A2 substance (KBr tablet).

Fig. 7 shows proton nuclear magnetic resonance spectrum of K01-0509-A2 substance (in deuterium oxide).

10 Fig. 8 shows carbon nuclear magnetic resonance spectrum of K01-0509-A2 substance (in deuterium oxide).

Best mode for carrying out the invention

The present invention will be explained by illustrating example, but the present invention is not limited within the
15 example.

Example

A loopful of the strain K01-0509 cultured by an agar slant medium (starch 1.0% (Wako Pure Chemical Industries Ltd., Japan), N-Z amine 0.3% (Wako Pure Chemical Industries Ltd., Japan), meat
20 extract 0.1% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), CaCO_3 0.3% (Kanto Chemical Inc., Japan) and agar 1.2% (Shimizu Shokuhin K.K., Japan), adjusted at pH 7.0) was inoculated into a medium (100 ml) (starch 2.4% (Wako Pure Chemical Industries Ltd., Japan), glucose 0.1% (Wako Pure Chemical
25 Industries Ltd., Japan), peptone 0.3% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), yeast extract 0.5% (Oriental Yeast Co., Ltd., Japan) and CaCO_3 (Kanto Chemical Inc., Japan), adjusted at pH 7.0) in a 500-ml Erlenmeyer flask and cultured at 27°C for 3 days by using rotary shaker (210 rpm) to obtain

seed culture liquid. The seed culture (200 ml) was inoculated into a production medium (20 liters) (starch 2.4% (Wako Pure Chemical Industries Ltd., Japan), glucose 0.1% (Wako Pure Chemical Industries Ltd., Japan), peptone 0.3% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), meat extract 0.3% (Kyokuto Pharmaceutical Industrial Co., Ltd., Japan), yeast extract 0.5% (Oriental Yeast Co., Ltd., Japan), calcium carbonate 0.4% (Kanto Chemical Inc., Japan), iron sulfate 7 hydrate $5.0 \times 10^{-4}\%$ (Kanto Chemical Inc., Japan), magnesium chloride 4 hydrate $5.0 \times 10^{-4}\%$ (Wako Pure Chemical Industries Ltd., Japan), copper sulfate 5 hydrate $5.0 \times 10^{-4}\%$ (Kanto Chemical Inc., Japan) and cobalt chloride 6 hydrate $5.0 \times 10^{-4}\%$ (Wako Pure Chemical Industries Ltd., Japan)) in a 30-L jar-fermenter, and cultured at 37°C for 4 days.

The medium cultured for 4 days (a total of 54 liters) was centrifuged by using Sharpless centrifuge to separate into the supernatant and microbial cells. The supernatant was charged on a column of active carbon ($\phi 75 \times 150$ mm, Wako Pure Chemical Industries Ltd., Japan), washed with water (1.5 liter), eluted the active principle with 20 and 40% acetone (each 1.5 liter), concentrated in vacuo and lyophilized. The obtained crude substance (21.5 g) dissolved in a small amount of water was charged on a column of Amberlite IRC-50 (H+) ($\phi 46 \times 110$ mm, Organo Corp., Japan), washed with water (300 ml). The active principle was eluted with 1N HCl (600 ml), neutralized, desalted by means of electrodialysis, concentrated in vacuo and lyophilized to obtain crude extract (823 mg).

The crude extract (200 mg) was dissolved in a small amount of water, and charged on an ODS column ($\phi 10 \times 30$ mm, Senshu

Scientific Co., Ltd., Japan) equilibrated with 0.1% aqueous trifluoroacetic acid solution, washed with 0.1% aqueous trifluoroacetic acid solution (10 ml), eluted the active principle with 20% methanol in 0.1% aqueous trifluoroacetic acid solution (5 ml), concentrated in vacuo and lyophilized. The remained crude substance (623 mg) was treated by the same manner to obtain active substance (103 mg). The active substance (103 mg) was dissolved in a small amount of water, and was purified by using the preparative HPLC (column: Develosil C30-UG-5, ϕ 20 × 250 mm, Nomura Chemical Co., Ltd., Japan). The UV absorption at 210 nm was monitored in the isocratic mobile phase of 8% methanol in 0.1% aqueous trifluoroacetic acid solution at flow rate of 5 ml/min. A peak showing activity at the retention time of 88 min. was observed and collected. The collected solution was concentrated in vacuo and lyophilized.

The obtained active material (8.3 mg) was dissolved in a small amount of water and purified by using the preparative HPLC (column: Develosil C30-UG-5, ϕ 20 × 250 mm, Nomura Chemical Co., Ltd., Japan). The UV absorption at 210 nm was monitored in the isocratic mobile phase of 3% acetonitrile in 0.05% aqueous phosphoric acid solution at flow rate of 5 ml/min. Peaks showing activity at the retention times of 27 min. and 30 min. were observed and collected. The collected solution was concentrated in vacuo, and charged on the ODS column (ϕ 5 × 10 mm, Senshu Scientific Co., Ltd., Japan) equilibrated with 0.1% aqueous trifluoroacetic acid solution, washed with 0.1% aqueous trifluoroacetic acid solution (5 ml), eluted the active substance with 100% methanol with 0.1% (final concentration) trifluoroacetic acid (5 ml) concentrated in vacuo and ly-

ophilized to obtain white powdery K01-0509-A1 substance (1.1 mg) and K01-0509-A2 substance (1.5 mg).

Industrial applicability

5 As explained hereinabove, the microorganism represented by the strain K01-0509, having ability to produce K01-0509-A1 substance and K01-0509-A2 substance, belonging to genus Streptomyces is cultured in a medium, and K01-0509-A1 substance and K01-0509-A2 substance having inhibitory activity against
10 type III secretion system are isolated. These substances can be expected as the selective and effective pharmaceuticals for treatment or prevention of infection with enteropathogenic gram negative bacteria having type III secretion system.